

Genome Mining and Transcriptional Analysis of Bacteriocin Genes in *Enterococcus faecium* CRL1879

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Abstract

Among 151 bacterial isolates from nine artisanal cheeses, *Enterococcus faecium* CRL 1879 showed antibacterial activity against the food-borne pathogen *Listeria monocytogenes*. The isolate produced a proteinase K-sensitive compound in the cell free supernatant. Genome analysis demonstrated the presence of enterocin A, enterocin B, enterocin P, enterocin SE-K4-like and enterocin X biosynthetic gene clusters. Nucleotide sequences encoding for a putative two-component bacteriocin were detected using bioinformatics tools, here named enterocin CRL1879αβ. A transcriptional analysis of all bacteriocin genes by quantitative real time PCR analysis (qRT-PCR) revealed the transcription of each enterocin gene at different levels. Finally, analysis of bacteriocin genes distribution in 251 *E. faecium* bioprojects was performed and compared to those identify in *E. faecium* CRL1879. The discriminative analysis demonstrated that bacteriocin genes are widely distributed among *Enterococcus*, independently of the origin of the strain.

The results presented in this paper represent a unique finding since this is the first demonstration of an *E. faecium* strain isolated from an artisanal cheese with the complete genetic machinery to produce six classes II and one class III bacteriocins.

Keywords: Bacteriocins; *Enterococcus faecium*; Genomes; Transcriptional analysis

Introduction

Enterococci are Gram-positive bacteria that commonly inhabit the gastrointestinal-tract of healthy humans and other animals. They are also associated with fermented foods, especially with dairy products such as cheese, where they are involved in flavor and aroma development. Several studies described the genus *Enterococcus* as the major component of the microbial community of certain Mediterranean and Hispanic cheeses [1-3].

Enterococci are known to produce a wide array of structurally diverse antimicrobial peptides, the so-called “enterocins”, which may contribute to improve the hygienic and safety properties of food products [4]. Such attractive traits have increased the interest for their biotechnological use in food preservation, especially since these compounds are particularly active against food-borne pathogens, such as *Listeria monocytogenes*. Artisanal products and traditional fermented milk products were reported to be excellent sources for the isolation of bacteriocin-producing lactic acid bacteria (LAB) [5-7].

Bacteriocins are antimicrobial peptides produced by bacteria, which are active against closely related species. Various classifications have been reported so far based on features such as mode of action, primary structure, presence of post-translational modifications or specific anti-bacterial activity [8,9]. The structural gene of a typical bacteriocin might be part of one to three operon-type structures being usually small. In addition, these operons also contain genes encoding for proteins involved in transport, regulation and/or immunity [10].

Classical methods used to characterize LAB bacteriocins are based on the purification, determination of the molecular mass and sequencing of the potential antimicrobial peptide present in the cell-free supernatant of the bacteriocin producer strain. However, the nature of bacteriocins entails a difficult and expensive purification process. Only a few reports have focused on the *in silico* genomic screening of open reading frames (ORFs) encoding for potential small peptides as a new

strategy to search for novel antimicrobial peptides [11,12]. In some cases, novel bacteriocin-related genes have been identified in LAB genomes [13,14]. Moreover, bacteriocin related genes in the genomes of non bacteriocin-producing strains have also been found [13].

In the present paper we described a promising *Enterococcus faecium* strain (CRL1879) isolated from an artisanal cheese, which displayed a remarkable antibacterial activity against the food-borne pathogen *Listeria monocytogenes*. The isolate produced a proteinase K-sensitive compound in the cell free supernatant. Genomic approaches were used to *in silico* characterize six class II bacteriocin clusters in this strain. Transcriptional analysis of all structural bacteriocin genes detected was performed in order to validate genomic finding. In addition, a careful analysis of the distribution, the presence and type of bacteriocin genes was evaluated in 251 *E. faecium* bioprojects and compared with those identify in *E. faecium* CRL1879. These data was used to subsequently examine if a set genes would be effective in predicting a category membership meaning if specific bacteriocin genes were present in niche-specific strains.

This is the first description of a food-related *E. faecium* (CRL1879) strain harboring six class II and one class III bacteriocin genes. The data presented here provides critical starting points for future functional studies on enterococcal bacteriocins.

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Materials and Methods

Source and maintenance of the cultures

E. faecium CRL1879 was isolated from an artisanal cheese of Northwestern Argentina and it is deposited under CERELA Culture Collection. *Listeria innocua* 7 (Unité de Recherches Laitières et Génétique Appliquée, INRA France); *L. monocytogenes* FBUNT (Bacteriology Department, Faculty of Biochemistry, National University of Tucumán); *L. monocytogenes* 24, *L. monocytogenes* 139, *L. monocytogenes* 149, *L. monocytogenes* 152 (Department of Dairy and Fat Technology, Institute of Chemical Technology, Prague); *L. monocytogenes* EGD-e [15], *E. coli* DH10 B (Invitrogen); *Staphylococcus (S) aureus*. ATCC29740. *E. faecium* ATCC19434; *E. mundtii* CRL 35 [6], *E. faecium* CRL1, CRL6, CRL11, CRL23, CRL147, CRL316, CRL318, CRL486, CRL589, CRL1571 (CERELA Culture Collection) were used in this study. All enterococci strains were routinely cultivated in LAPTg (peptone, 15 g/l; tryptone, 10 g/l; yeast extract, 10 g/l; glucose, 10 g/l; Tween 80, 0.1 % v/v; agar 1.5%) at 37°C. *Listeria* and *Staphylococcus* strains were grown in complex media Brain Heart Infusion broth (BHI, Britania, Buenos Aires, Argentina) at 37°C and *E. coli* DH10 B in LB medium [16] at 37°C under aerobic conditions. All cultures were grown overnight. Bacterial stock cultures were maintained in 10% (w/v) skim milk with 0.5 % (w/v) yeast extract and 10 % (v/v) glycerol at -80°C. Agar plates were prepared using each culture medium plus 1.5% of agar (Britania, Buenos Aires, Argentina).

Antimicrobial activity assays of *E. faecium* CRL1879

Cell-free supernatant (CFS) of *E. faecium* CRL1879 was screened for antimicrobial activity by the agar diffusion assay [17] against the indicator strains. Briefly, CFS was obtained by growing *E. faecium* CRL1879 in LAPTg broth for 16 h at 37°C. The bacterial culture was centrifuged at 15.600 × g for 10 min to obtain CFS. Then 5 µl of CFS was spotted in plates containing 10 ml of BHI 1.5% agar plus 10 ml of BHI soft agar (0.7%) inoculated with 10⁷ colony forming unit.ml⁻¹ of an overnight culture of the indicator strains. Plates were incubated for 24 h at the corresponding incubation temperature and examined for clear zones of growth inhibition. In order to characterize the antimicrobial compound, aliquots of 200 µl of CFS were a) adjusted to pH 7.0 with 1 N NaOH and b) added 1000 U ml⁻¹ of catalase, c) treated with proteinase K (20 mg.ml⁻¹-Invitrogen, Buenos Aires, Argentina) at 42°C for 2 h, d) heated at 100°C for 5 min (most bacteriocins are heat stable). In all the cases, a positive control (fresh unmodified CFS) was tested in parallel. The inhibitory activity of each sample was determined by the spot-on-lawn assay as described above.

In silico identification of bacteriocin genes in *E. faecium* CRL1879 genome

E. faecium CRL1879 genome sequence is deposited at Genbank (AN AOUK000000000.1). Genes encoding bacteriocins and post translationally modified peptides (RiPPs) or those related with their production systems were search in *E. faecium* CRL1879 genome. Rapid Annotations using Subsystem Technology server (RAST) and BAGEL3 software Linux version were used to perform the analysis [18,19]. Putative promoter regions were analyzed with Promoter Prediction by Neural Network (NNPP) and BPROM (Softberry Inc., Mount Kisco, NY, USA) [20]. Bacteriocin sequence analyses were done with the bacteriocin database [21,22]. The analysis of the nucleotide sequences, translation and alignments were performed with DNASTAR software version 11.2.1.25 (DNASTAR Inc., USA). The identity of sequences was analyzed using *Blastx* service from the NCBI database.

Transcriptional analysis of bacteriocin genes in *E. faecium* CRL1879

RNA isolation: Total RNA was extracted from *E. faecium* CRL 1879 according to Raya et al. from three independent cultures and separated into at least two technical repeats [23]. Briefly, cells from 50 ml of a culture of 12 h were harvested by centrifugation, washed with TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and suspended in 500 µl of cold TE buffer. The cell suspension was added to a 2-ml screw-cap microcentrifuge tube containing 0.6 g of glass beads (0.1-mm diameter), 170 µl of 2% macaloid slurry, 500 µl of Tris-HCl buffered phenol-chloroform (1:1), and 50 µl of 20% sodium dodecyl sulfate. Cells were disrupted by shaking in a Mini-Beadbeater Cell Disrupter (Model 607EUR, Biospec Products, Bartlesville, Okla, USA) during 6 cycles of 1min of disruption following by 1 min on ice. After centrifugation at 15.600 × g, 4°C for 15 min, the aqueous supernatant containing the RNA was two times extracted with 1volume of phenol-chloroform and precipitated with sodium acetate 3 M, pH 5.2 and absolute ethanol. Finally, RNA was suspended in RNase free water. The isolated RNA was treated with TURBO DNA-free™ kit (Ambion®, Invitrogen, Buenos Aires, Argentina) according to the manufacturer's instructions, before the first-strand synthesis of cDNA.

qRT- PCR: Synthesis of cDNA were performed from 100 ng of total DNA-free RNA, using qScript™ cDNA SuperMix (Quanta-Biosciences, Genbiotech, Argentina). The primers used in qRT-PCR are listed in (Table 1). These were designed according to the *entA*, *entB*, *entP*, *ent entSE-K4*, *entXaβ* and *entCRL1879aβ* sequences according to the genome sequence of *E. faecium* CRL1879 with Primer Quest software (Integrated DNA Technologies, Inc.). [26]. qRT-PCR reactions were performed in duplicates using 35 ng cDNA, 0.3 µM of each primer and 10 µl of qPCR MasterMix (PerfeCta® SYBR® Green Supermix for iQ™, Quanta Biosciences™, Genbiotech, Argentina) on a final volume of 20 µl per reaction. Reactions were run on an IQ™ 5 Multicolor Real-Time PCR Detection System (BioRad, USA). PCR conditions were: initially 2 min/50°C, 3 min/ 95°C, 40 cycles consisting of 10 s/ 95°C, 30 s/50°C following by a melting curve temperature cycles. For each reaction, a non-template control (NTC) was included (without cDNA) and the invariable expression of 16S rRNA under the test conditions (data not shown) was used as house-keeping gene. Standard curves for *entA*, *entB*, *entP*, *entSE-K4*, *entXaβ* and *entCRL1879aβ* genes were performed in order to determine the copy number of each transcript. These genes were amplified by PCR from *E. faecium* CRL 1879 genomic DNA and purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison-USA). All primers and standard curves for qRT-PCR assay displayed efficiencies between 98 and 110%. A single product-specific melting curve was obtained for each primer set. The concentration of the amplified product was measured with a UV-visible spectrophotometer (Varian, 50MPR Microplate Reader -Cary) at 260 nm using a standard calibration curve, and converted to number of copies per unit volume using the following equation:[25].

$$\text{DNA (copy)} = (6.023 \times 10^{23} [\text{copies mol}^{-1}] \times \text{DNA amount [g]}) / (\text{DNA length [bp]} \times 660 [\text{g mol}^{-1} \text{ bp}^{-1}])$$

The concentration of the purified linear dsDNA standards was adjusted to 10¹⁰ copies per µl. This stock solution was tenfold serially diluted to obtain a standard series from 10¹⁰ to 10⁴ copies per µl and it was used to construct the standard curves for enterocin structural genes. Each standard dilution was analyzed in duplicate. The Ct values were plotted against the logarithm of their initial template copy concentration to generate each standard curve by a linear regression.

Primers	Nucleotide sequence	Product size (bp)	Reference
Enterocin A	F: 5' AATATTATGGAATGGAGTGATTGC 3' R: 5' TTGCCTTGGCCCAATCGACC 3'	68	This study
Enterocin B	F: 5' CAGAATGCCTAATGAGTTAAATAGACC 3' R: 5' AGAGTATACATTTGCTAACCCAGC 3'	138	
Enterocin P	F: 5' GATGCAGCTACGCGTTCATATGGT 3' R: 5' CAAACCAGAAGCCAGCCACTAAT 3'	98	
Enterocin SE-K4	F: 5' GGCTCAGTTATTTGTAGCATGATTGGG 3' R: 5' CACACCATTTCGTAATACGTGGC 3'	86	
Enterocin CRL1879 α	F: 5' CTGGCTGGGATGCTCGAA 3' R: 5' GTGGACTTACCACAGGGGCA 3'	128	
Enterocin CRL1879 β	F: 5' GGATTCAGCATGTGGACCGG 3' R: 5' GGCACTACTTTTCTGGGGC 3'	121	
Enterocin Xα	F: 5' ACTTCTACAGATTCAGGAGGAATAG 3' R: 5' CTGAGTGATCCCATGCATCTT 3'	93	
Enterocin Xβ	F: 5' CTTTGGTATGGGTAGGACAATTTATG 3' R: 5' CCAGGAATTATCATGTGTTTAACAGG 3'	93	
16s rDNA	F: 5' ATTGCCGAAGATTCCCTACTG 3' R: 5' GCATTAGCTAGTTGGTGAGGT 3'	135	

Table 1: qRT-PCR specific primers used in this study.

Sensitive strains	Antimicrobial Activity (mm)	
	ATCC	CRL 1879
	19434	
<i>E. coli</i> DH10 B	-	-
<i>E. faecium</i> ATCC19434	-	-
<i>E. faecium</i> CRL1	-	-
<i>E. faecium</i> CRL6	-	++
<i>E. faecium</i> CRL11	-	++
<i>E. faecium</i> CRL23	-	+
<i>E. faecium</i> CRL147	-	-
<i>E. faecium</i> CRL316	-	+
<i>E. faecium</i> CRL318	-	++
<i>E. faecium</i> CRL486	-	++
<i>E. faecium</i> CRL589	-	++
<i>E. faecium</i> CRL1571	-	++
<i>E. mundtii</i> CRL35	-	+
<i>L. innocua</i> 7	+	+
<i>L. monocytogenes</i> 139	+	+
<i>L. monocytogenes</i> 149	+	+
<i>L. monocytogenes</i> 152	+	+
<i>L. monocytogenes</i> 24	+	+
<i>L. monocytogenes</i> EGD-e	-	-
<i>L. monocytogenes</i> FBUNT	+	++
<i>S. aureus</i> ATCC29740	-	-

Diameter of growth inhibition zones (mm) (++) : >10 mm; (+) : <10 mm; (-): no growth inhibition zone.

Table 2 Inhibitory activity of heated and neutralized cell-free supernatants of *E. faecium* CRL1879 and ATCC 19434 strains as determined by the agar spot test method.

When assaying the samples for each enterocin expression, the corresponding standard series were run under the same conditions and the copy numbers of samples was determined by reading off the standards series with the Ct values of the samples.

Genomic mining of putative bacteriocin genes in *E. faecium* bioprojects

251 *E. faecium* genomes bioprojects available at NCBI Genome

database were analyzed by BAGEL3 software Linux version, BLASTx algorithm and bacteriocin database Bactibase [20-23] (Table S1). The resulting data from BAGEL3 was subjected to linear discriminant analysis (LDA). LDA was used to establish whether the presence of a set of *bac* genes was effective in predicting category membership, i.e, specific *bac* genes present in niche-specific strain. LDA was performed with Statistica 10.0 software. The discrete distribution of *bac* genes among strains isolated from human, animal, food and those of unknown provenance was determinate. All genomes analyzed were previously classified depending on the origin of isolation of the strains, i.e, animal (AN); human (H); food (F) sources and strains with unknown provenance (U) (Table S2).

Results

Antibacterial activity of *E. faecium* CRL1879

E. faecium CRL1879 was isolated from an artisanal cheese made in Northwestern region of Argentina. This strain was selected due to the interesting antimicrobial activity observed in cell free supernatant (CFS); *E. faecium* ATCC19434 (enterocin A and B producer, unpublished data) was used as positive control (Table 2). All *L. monocytogenes* strains, including biofilm-forming strains 24,139,149,152, were sensitive to CFS except EGD-e strain. Regarding enterococci used as indicators, it is surprisingly that CFS from CRL1879 turned out to be active against almost all strains tested as compared to the antimicrobial activity of the ATCC19434 CFS, suggesting the potential production of different antimicrobial compounds among strains. On the other hand, CFS did not show antimicrobial activity against *E.coli* DNH10B, *S. aureus* ATCC 29740. To further characterize the antimicrobial compound, CFS aliquots were i) neutralized to pH 7, ii) treated with catalase and iii) with proteinase K and tested against *L. monocytogenes* FBUNT. Untreated CFS from CRL1879 was used as positive control. Growth inhibition halos were only sensitive to proteinase k demonstrating that the antimicrobial compound present in CFS was of proteinaceous nature, likely a bacteriocin.

Genome identification of bacteriocin production systems in *E. faecium* CRL 1879

The genome sequence of *E. faecium* CRL 1879 was acquired using a whole-genome shotgun (WGS) strategy with an Ion Torrent personal genome machine. Quality filtered reads were *in silico* assembled

using the DNASTAR Ngen assembler, giving 73 large contigs (AN AOUK0000000.1) [24]. RAST Server, Blast algorithms and BAGEL3 analysis demonstrated the existence of enterocin P, enterocin B, enterocin X $\alpha\beta$, enterocin A and enterocin SE-K4-like biosynthetic clusters (Figure 1).

Sequence analysis of the enterocin P gene cluster (contig_152, AN AOUK01000025) revealed the same genetic organization as previously described by Cintas et al. [26] (Figure 1). The deduced amino acid sequence of *entP* gene showed 95% identity to enterocin P described by Cintas et al. with two amino acids changes in the leader peptide at positions 9 (A-T) and 13 (I-K) and one at the position 69 (M-I) of the mature peptide [26].

Enterocin B biosynthetic cluster from *E. faecium* CRL1879 (contig_71, AN AOUK01000001) was identical to the one previously described by Franz et al. [27]. By using the software BAGEL 3, genomic sequence analysis of the same contig revealed the presence of ORFs encoding for two -component bacteriocins, enterocin X previously described in *E. faecium* KU-B5 [28]. A putative immunity gene for enterocin X (*entiX*) was located 584 nucleotides upstream of the enterocin B precursor gene, in the same direction. The putative two components of enterocin X locus containing enterocin X α (*entX α*) and the enterocin X β genes (*entX β*) was located upstream *entiX* (Figure 1).

BLAST and RAST analysis of contig_191 (AN AOUK01000051) demonstrated that a fragment of 700 bp display 94% of identity with *E. faecalis* pAMS1 (NG_036515.1) and 92% with pEK4S (AB092692.1) plasmids, both known for their bacteriocin-encoding genes [29,30]. The biosynthetic cluster of this enterocin SE-K4-like bacteriocin is organized in an operon-like structure consisting in two consecutive ORFs (Figure 1). The first ORF 1 encodes a 71 amino acid protein corresponding to enterocin SE-K4-like pre-peptide. The deduced amino acid sequence of the putative bacteriocin encoding gene in CRL1879 genome exhibited only 77% of identity to enterocin SE-K4 first described in *E. faecalis* K4 (BAC20326.1) with six amino acid deletion at the C-terminal domain (Figure 1). However, this nucleotide sequence is widely distributed and conserved among *E. faecium* genomes strains deposited at NCBI (EEV57603.1, EFF32511.1, EFR67967.1, EJX61666.1, EJX72841.1, among others). ORF2 gene, encoding a putative immunity protein composed of 113 amino acids, was immediately found downstream of the bacteriocin gene. A putative ribosome binding site (RBS) for the bacteriocin structural gene (AAGGTG) was located 7 bases upstream of the initiation codon. Possible -10 and -35 promoter sequences were also detected.

The enterocin A locus was located in contig_279 (AN AOUK01000059) (Figure 2). Sequence analysis showed the presence of two putative operons: (I) the bacteriocin operon with the enterocin A gene (*entA*), the immunity gene (*entI*), and three component regulatory system the peptide pheromone gene (*entF*), the histidine protein kinase gene (*entK*), and the response regulator (*entR*), and (II) the transporter operon consisting of the two genes, the ABC transporter (*entT*) and its accessory gene (*entD*) as previously described by O' Keffee et al. [31]. Analysis of 1474 bp intergenic sequence located between enterocin A-regulatory and transport operons revealed three consecutive ORFs oriented in opposite direction to the complete *entA* cluster. Both, ORF 1 and ORF2, encode putative double-glycine-type pre-peptide with a 19 and 21-residue leader sequence with theoretical pI/MW 9.31/5141.86 and 7.78/4338.99, respectively (Figure 2). Although deduced amino acid sequence of ORF1 showed 97- 99% identity with annotated as putative bacteriocin-like proteins present in several *E. faecium* genomes (YP_008396028.1; YP_007395047.1; EEV45206.1;

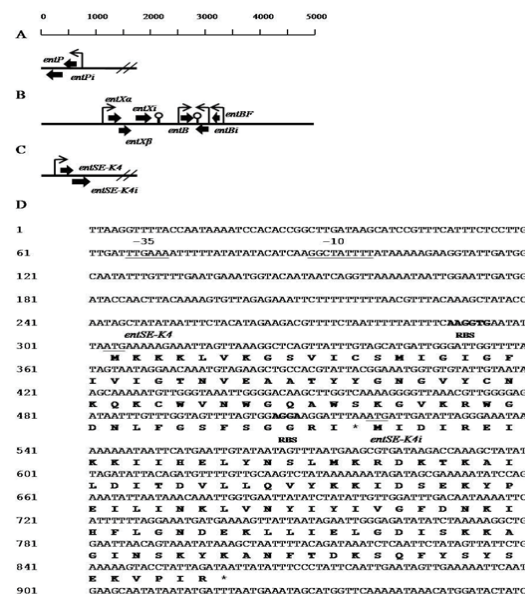


Figure 1: Genetic organization of gene clusters associated with enterocin production in *E. faecium* CRL 1879 draft genome. Graphic illustration of the genes involved in the production, immunity and transport of enterocin P (A), enterocin X and enterocin B (B); enterocin SE-K4 (C) are represent. Potential transcriptional terminators structures are indicated by a stem-loop vertical line and promoters are indicated by an arrow. (D) Nucleotide sequence of the enterocin SE-K4 gene and of the putative immunity protein gene. The translation products of both genes are also shown. The putative -10 and -35 promoter sequences are underlined; ribosome binding sites (RBS) are boldface.

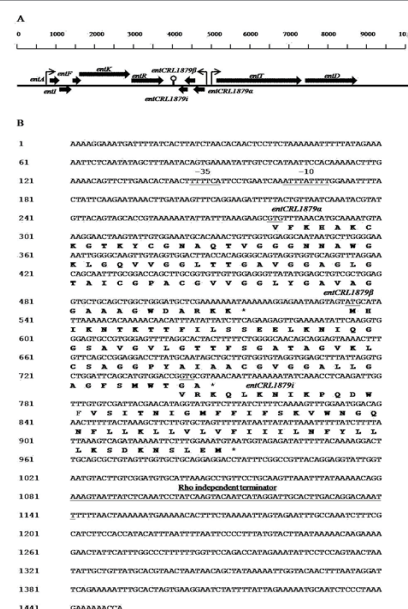


Figure 2: Genetic organization of enterocin A and enterocin CRL1879 $\alpha\beta$ gene cluster in *E. faecium* CRL 1879 draft genome (A). Potential transcriptional terminators structures are indicated by a stem-loop vertical line and promoters are indicated by an arrow. (B) Nucleotide sequence of the 1450-bp fragment containing both enterocin CRL1879 structural genes (*ent1879 α* , *ent1879 β*) and the putative immunity gene (*orf3*) is represent. The deduced amino acid sequences of enterocin CRL1879 and ORF3 are shown. The putative -10 and -35 promoter sequences and Rho independent terminator sequence are underlined.

EEF20746.1), no clear homology was found to any biochemically characterized bacteriocin. ORF2 deduced amino acid sequence showed 97-99% identity with hypothetical proteins (YP_007395046.1; YP_008396027.1; EEV45207.1) but no homology to any previously known bacteriocin. Sequence analysis of the deduced amino acid sequence of these two putative peptides also showed the GXXXG motif, suggested to be involved in helix-helix interaction between two component bacteriocins [32]. No obvious *rho*-independent transcriptional terminator (invert repeats) was found between ORF1 and ORF2 suggesting that may belong to the same transcriptional unit. Thus, we named here enterocin CRL1879αβ as a potential two-component bacteriocin. In addition, ORF3 located downstream of ORF2 predicted a 63-residue, hydrophobic and cationic (pI 10.47) protein with one putative trans-membrane domain resembling an immunity-like protein, but no sequence identity could be determined either.

E. faecium CRL 1879 expresses multiple bacteriocin genes

In order to validate genomic findings we performed transcriptional analyses of all putative bacteriocin genes found in *E. faecium* CRL 1879. qRT-PCR experiments revealed the presence of *entP*, *entSE-K4*, *entB*, *entA*, *entXαβ* subunits as well as *entCRL1879αβ* transcripts (Table 3). The gene encoding for the enterocin P structural peptide presented the highest level of expression (2.4×10^7 copies μl^{-1}), while enterocin Xα and Xβ gene transcripts were present in an order of 1.6×10^6 and 2.7×10^6 copies μl^{-1} respectively. The *entA* gene was transcript in an order of 2.4×10^5 copies μl^{-1} . Interestingly, *entCRL1879αβ* genes were transcript at 7.7×10^5 and 7.4×10^5 copies μl^{-1} , *entSE-K4* transcript was present at 2.5×10^5 copies μl^{-1} and finally *entB* transcript was in an order of 9.2×10^4 copies μl^{-1} .

Comparative genomic analysis of putative bacteriocin genes among *E. faecium* strains

A detailed *in silico* analysis of the distribution, the presence and type of *bac* genes was evaluated in *E. faecium* bioprojects and compared with those identify in *E. faecium* CRL1879. 251 partial or complete *E. faecium* genomes, deposited at the NCBI Genome database, from

different origin were mine searching bacteriocin genes using BAGEL3 software and BLASTx algorithm (Table S1). Exhaustive analysis demonstrated that i) almost all strains contain one to six putative bacteriocin genes except to PRJNA82503 and PRJNA179542 which exhibited no putative bacteriocin genes ii) 24 bacteriocin class II encoding genes and one for enterolysin A, a class III bacteriocin, were detected; iii) known enterocins (enterocin A, B, P SE-K4, X, NKR-5-3A, L50A and B) showed a highly conserved sequence among the genus; iv) not all strains carrying enterocin A genes have enterocin B gene v) enterolysin A, a bacteriolysin, is widely distributed among *E. faecium* genomes; vi) as a general rule, enterocin Xαβ is disseminated among *E. faecium* strains that concomitantly harbors *entB* gene. One strain showed enterocin Xαβ in the absence of *entB* gene. Conversely, six genomes showed only *entB* gene; vii) interestingly, we found genes encoding for bacteriocins described in the Lactobacillus genus (sakacin Q and sakacin T) and viii) *E. faecium* CRL1879 is the only food related strain harboring six class II and one class III bacteriocin genes. The resulting data from BAGEL3 was subjected to LDA statistical analysis. Each individual strain was *a priori* classified according to its origin of isolation (Table S1). Once each bacteriocin gene was detected by BAGEL3, LDA was used to establish whether the presence of a set of *bac* genes was effective in predicting category membership i.e. the origin of a strain (Figure3) (Table S2). This analysis measure the percentage of similarities according to the presence or absence of variables used to identify each individual. Results demonstrated that 53% of strains analyzed were classified in the same groups as the *a priori* classification (Table 4). LDA showed that 63.33% of total strains *a priori* classify as Human could be assigned in the same group. However, the discriminative analysis demonstrated bacteriocin genes are widely distributed among *Enterococcus*, independently of the origin of the strain and there is no strong association between the origin and the number of bacteriocin genes present in a specific strain.

Discussion

Northwestern Argentinean chesses have been prolific substrates where numerous LAB strains with technological properties were isolated and characterized [17]. Several studies described the genus

<i>E. faecium</i> CRL1879	Ct and estimated enterocin copy number by RT-PCR absolute quantification*							
	Bacteriocin mRNA copies (copies μl^{-1})							
	<i>entA</i>	<i>entB</i>	<i>entP</i>	<i>entSE-K4</i>	<i>entXα</i>	<i>entXβ</i>	<i>ent1879α</i>	<i>ent1879β</i>
	2.4×10^5	9.2×10^4	2.4×10^7	2.5×10^5	1.6×10^6	2.7×10^6	7.7×10^5	7.2×10^5

*Estimated copy number of enterocin genes transcripts in *E. faecium* CRL 1879 cells at laboratory conditions, by RT-PCR absolute quantification.

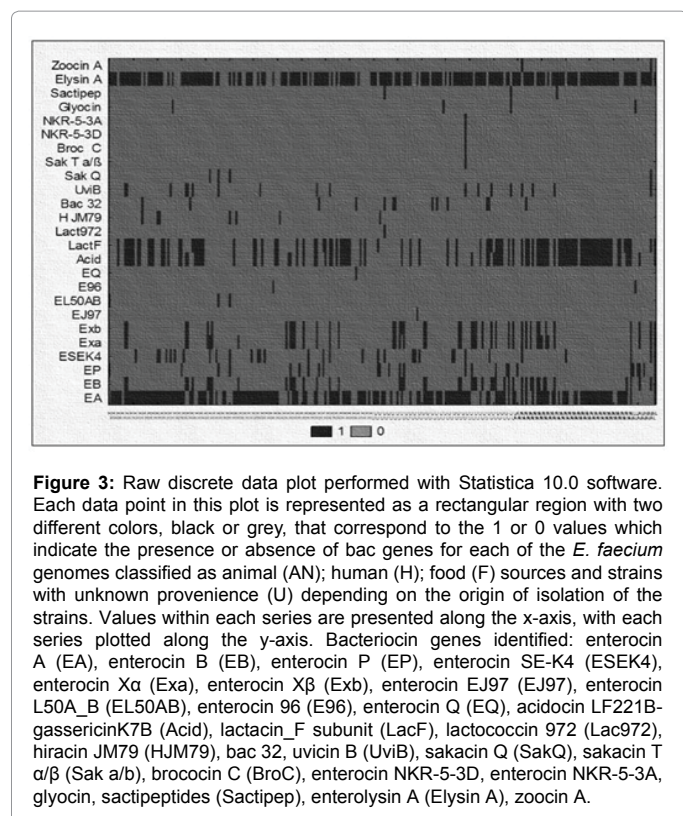
Table3: Enterocin transcripts copy number obtained by qRT-PCR.

<i>A priori</i> classification*	% of strains defined in the corresponding category classification**	Classification Matrix			
		Predicted Category Classifications by LDA			
		Human (H)	Unknown (U)	Animal (AN)	Food (F)
H	63.6	77	11	28	5
U	26.9	34	18	8	7
AN	65.4	14	2	34	2
F	45.4	4	2	0	5
Total	53.4	129	33	70	19

*: *A priori* classification according to the strain isolation source information obtained from the Bioproject at the NCBI Genome data base.

**:: Percentage of strains that effectively correspond to their *a priori* established classification after the LDA analysis.

Table 4: Predicted category classifications of *Enterococcus* strains harboring bacteriocin genes by LDA.



Enterococcus as the major component of the microbial community of certain Mediterranean and Hispanic cheeses [2,3,33]. The predominance of enterococci into the microbial population of various fermented foods such as meat or cheese can be explained by the frequent production of bacteriocins, as well as its tolerance to heat, dry and saline environments [34,35]. In the present paper, we demonstrated that CFS of CRL1879 strain is active against six different *Listeria* strains including biofilm-forming bacteria (strains 139, 149, 152, 24) except to the widely used *L. monocytogenes* EGD-e. In contrast, CFS was not active against *E. coli* DNH10B neither to *S. aureus* ATCC 29740 used as indicator strains. In general, bacteriocins produced by Gram-positive bacteria have no effect on Gram-negative bacteria without the addition of any membrane-active compound, but there is some exceptions to the rule [36].

E. faecium CRL1879 strain have known structural genes encoding for class II bacteriocins: enterocin A, enterocin B, enterocin P, enterocin X and enterocin SE-K4-like. Enterocin A and B first discovered and identified in *E. faecium* T136 are frequently present in *E. faecium* strains from various sources [37,38]. Enterocin P is a sec-dependent bacteriocin widely distributed among *E. faecium* strains. We have previously shown that the presence of more than one bacteriocin gene in *Enterococcus* genus is a common finding [39]. Interestingly, the gene encoding for enterocin SE-K4 was only described in *E. faecalis* strains [29]. The co-existence of genes encoding for the production of enterocin SE-K4-like bacteriocin with other *bac* genes in *E. faecium* strains have not been described in the literature yet. To our knowledge, this is the first report of an *E. faecium* strain that possess the structural gene encoding for enterocin SE-K4-like bacteriocin.

Since the first enterococcal genome sequenced - that of *Enterococcus faecalis* V583, now a veritable avalanche of complete or draft genome

sequences of various enterococcal strains and species are available [40]. Recently as part of this paper, *E. faecium* CRL1879 genome has been sequenced by a whole-genome shotgun [24]. Based on these data, *in silico* screening strategies were employed to mine for bacteriocins or ORFs encoding for potential small peptides in DNA sequences using specially designed software such as BAGEL [20]. Genome sequence analysis of *E. faecium* CRL1879 confirmed the presence of gene clusters involved in enterocin P, B, A and SE-K4-like bacteriocin production, transport and immunity. They are chromosomally encoded and they show high degree of similarity to those previously described in the literature except for enterocin SE-K4 as described in the results section. In addition, a gene cluster encoding for enterocin X production was also found. Enterocin X cluster was located in the vicinity of enterocin B in similar fashion as previously described in the literature [28]. BAGEL 3 analyses also revealed a potential two-component bacteriocin at the intergenic sequence located between enterocin A-regulatory and transport operons. Although, three consecutive ORFs (*ent* CRL1879 $\alpha\beta$ and CRL1879 $_{imm}$) oriented in opposite direction to the complete *entA* cluster are present in several *E. faecium* genomes, we demonstrated that *ent* CRL1879 $\alpha\beta$ are transcribed under our laboratory conditions (Table S2). Transcriptional analyses showed also that *entP* transcript showed the highest copy number which might be explained since enterocin P is exported via the general secretory (Sec-dependent) pathway. Such a mechanism of export is potentially beneficial to the host due to its apparent metabolic and genetic economy. Only a few studies have analyzed the transcriptional profile of the bacteriocin production in LAB. A reverse transcription-PCR analysis showed the expression of *entA*, *entB*, and *munKS* bacteriocin genes in *E. faecium* CWBI-B1430 and *entP* and *munKS* genes in *E. mundtii* CWBI-B1431 [41]. Moreover, enterococcal strains characterized in the literature so far bear up to four bacteriocin genes [42, 9]. qRT-PCR results obtained here validated the *in silico* bacteriocin genome findings demonstrating that the *E. faecium* CRL 1879 strain isolated from an Argentinean northwestern artisanal cheese possess the complete genetic machinery necessary to produce six antimicrobial peptides.

Enterococci are known to produce a wide array of structurally diverse antimicrobial peptides [26]. This fact was here validated by the number and type of putative bacteriocin genes found in enterococcal genomes by BAGEL3 analysis. Enterocin A and B were considered as *hallmark* genes of this genus; however we found that not all strains carrying enterocin A genes have enterocin B gene as previously described by other authors [43]. Another interesting fact is that we found that genes of enterocin X $\alpha\beta$ and its immunity protein are located in the vicinity of enterocin B gene cluster in *E. faecium* CRL1879. Genome-wide analysis revealed that enterocin X $\alpha\beta$ previously described in *E. faecium* KU-B5 is widely distributed among *E. faecium* strains that concomitantly harbors *entB* gene [28]. Exceptions to this rule are only six strains that contain only enterocin B gene. We also detected enterolysin A, a bacteriolysin, extensively distributed among *E. faecium* genomes besides its previous characterization only in *E. faecalis* strains [44]. In addition, we found genes encoding for bacteriocins described in the *Lactobacillus* genus such as sakacin Q and sakacin T besides those previously described in *Enterococcus*.

In summary, bacteriocin genes are extensively distributed among *Enterococcus* genus, independently of the origin of the strain and there is no strong association between the origin and the number of bacteriocin genes present in a specific strain. This is first demonstration of a bacterial strain, *E. faecium* CRL 1879, able to produce six class II bacteriocins. *E. faecium* CRL 1879 has a promising potential in food preservation. Ongoing research is evaluating the bacteriocin producing

machinery in a model food system.

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